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Supplementary Material for

Internalization of *Salmonella* by Macrophages Induces Formation of Nonreplicating Persisters

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Materials and Methods:

Bacterial strains and plasmids. The *S.* Typhimurium strains used in this study were wild- type 12023s and its mutant derivatives (Table S1). All *S.* Typhimurium strains used for fluorescence experiments harboured the pFCcGi (*3*), pDiGc or pDiGi FD plasmids (*4*) and fully aerated growth media (rich medium was Luria Bertani (LB) for Colony Forming Unit (CFU) assays and minimum medium was MgMES (*8*) for Fluorescence Dilution (FD) assays) were supplemented with 0.2% L-arabinose or 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to allow production of fluorescent proteins. The *E. coli* strain used was wild-type MG1655.

Bacterial mutagenesis. *S.* Typhimurium mutant strains were constructed using a one-step λ Red recombinase chromosomal inactivation system (*1*). Plasmids pKD3 or pKD4 were used as the template to amplify the chloramphenicol (cm) or kanamycin (km) resistance gene, respectively, and amplification reaction products were transferred into pKD46-containing bacteria expressing λ Red recombinase by electroporation (*1*). To verify that the phenotypes were not due to secondary mutations, independent mutagenesis was conducted (Fig. S9).

Mouse infections. Mice (6-12 week old, C57 Bl6, BALB/c or 129SV *slc11a*+) were inoculated intragastrically with 200 µL of 3% $NaHCO_3$ in phosphate buffered saline (PBS) containing 2 x 10^{10} CFU of single strain or combined wild-type and mutant strains (Competitive Index (CI)) at a ratio of 1:1. Bacterial inocula were prepared by 3 h sub-culture in rich LB medium to reach mid-exponential growth phase prior to mice gavage. When indicated, mice received enrofloxacin in drinking water (2 g/L). For experiments done in the absence of enrofloxacin treatment and where mice were sacrificed after 6 days p.i. (CI and Fig. 1D), 2 x 10^8 CFU were inoculated intragastrically. Mice were sacrificed at different times p.i. and Peyer's Patches (PP), Mesenteric Lymph Nodes (MLNs), and spleens were collected, homogenised using the plastic end of a 5 ml pipette in sterile petri dishes, then passed through the mesh of a 100 µm cell strainer (BD Falcon) in 20 ml of Lysis buffer (0.3% Triton X-100 in 20% PBS). Cell suspensions in Lysis buffer were vortexed vigorously to ensure efficient host cell lysis then centrifuged at 5,000 *g* for 10 min at 4°C. Bacterial pellets were resuspended in PBS and either plated for CFU enumeration or fixed in 1% paraformaldehyde (PFA) for 10 min at RT prior to Flow Cytometry analysis. For all CI experiments, at least 100 recovered bacteria were patched onto LB or LB containing km (50

μg/ml) plates to determine the relative proportions of WT and mutant strains.

Flow cytometry analysis. Samples were acquired on a FACSCalibur cytometer (Becton Dickinson (BD)) using Cell Quest Pro software or on a LSRFortessa cytometer (BD) using FACSDiva software. Fluorophores were excited at 488 nm, 532 nm or 561 nm, where appropriate. On the FACScalibur, green and red fluorescence were detected at 510/20 nm and 595/20 nm, respectively. On the LSRFortessa, green fluorescence was detected at 530/30 nm, red fluorescence at 610/20 nm. Data were analysed with FlowJo Software version 8.6.6. (TreeStar, Inc.). To analyse fluorescence dilution, bacteria were identified after gating on the constitutive fluorophore-positive signal. The extent of replication of the population (F, fold replication) was calculated by the ratio: Yo/Yt (where Y is the geometric mean of diluted fluorescence intensity of the bacterial population at a specific time) (*4*).

Live imaging microscopy. For visualization of regrowth of non-replicating bacteria isolated from MLNs of infected mice, microscope slides were prepared as described in (*2*). A total of 31 non-replicating bacteria was observed, out of which 30 resumed growth. For experiments to assess the effect of antibiotic on replicating and non-replicating bacteria, bone marrow-derived macrophages (BMM) were seeded onto 35 mm glass-bottom culture dishes (at a density of 1×10^5 cells per dish) (MatTek, Ashland, MA), 16–20 h before infection. Cells were infected with *S.* Typhimurium 12023 containing pDiGc as described in (*3*). Before imaging, cells were washed and then incubated in imaging medium (*6*) containing 100 μg/ml of cefotaxime. Bacteria were imaged using a Zeiss Axiovert 200 M microscope (Carl Zeiss, AG, Germany) (*6*). Volocity software (Improvision) was used for both recording and image processing.

Cell culture and BMM infection. Primary BMM were extracted from C57 Bl/6 mice (Charles River), C57 Bl/6 *gp91/phox-/-* mice or 129SV *slc11a+* mice, grown and infected as described in (*3*) with stationary phase bacteria grown overnight in fully aerated LB medium (CFU assays) or MgMES (FD assays) at an Multiplicity Of Infection (MOI) of 5-10. Gentamicin was not added to the infection medium with the exception of Fig. 2A (green kinetics), Fig. 2D, Fig. 4D and Fig. S7. Where indicated, BMM were pre-activated 16 h prior to infection with 0.1 μg/ml IFN-γ (Invitrogen) or pre-treated with 0.1 μg/ml Latrunculin B, 0.5 μM Cytochalasin D or 0.5 μM

Bafilomycin A1 resuspended in DMSO (Sigma), for 30 min prior infection. Measurement of BMM lysosomal Cathepsin B activity using Magic Red-RR was carried out as described in (*5*).

Persister assays. For quantification of persisters in LB or minimal medium stationary phase bacteria grown overnight in LB medium were inoculated directly (1/400 dilution) in fresh LB medium containing cefotaxime (100 μg/ml), ciprofloxacin (1 μg/ml), gentamicin (50 μg/ml) or different combinations thereof, and grown up to 24 h at 37°C with aeration. Where indicated, stationary phase bacteria from overnight cultures were pre-exposed to 8 mM Serine Hydroxamate (SHX) in LB medium or acidified LB medium (pH 4.5) for 30 min prior to transfer to fresh LB medium (pH 7.2) containing antibiotics. Macrophage-induced persisters were recovered as follows: after 30 min infection of BMM with stationary phase bacteria grown overnight in LB medium (*3*), BMM were lysed and intracellular bacteria collected, washed once in PBS and resuspended in fresh LB medium containing various antibiotics. The fold increase in persisters caused by internalization in BMM was quantified by calculating the ratio of percentage of survival of the BMM-exposed population over that of the same population used for the BMM inoculum, subsequently grown only in LB medium containing antibiotics (LB persisters).

Metabolic activity assays. GFP-induced *Salmonella* 12023 carrying pDiGi were used to infect BMM in the absence of IPTG to allow for GFP dilution. Cefotaxime (200 μg/ml) was added to the infection medium 30 min p.i. in the absence of gentamicin to kill replicating bacteria. After 24, 48 or 72 h of infection, arabinose was added to the infection medium and DsRed was measured 4 h later by Flow Cytometry. To measure regrowth of non-replicating bacteria, BMM were lysed to release bacteria; these were washed once in PBS prior to inoculation into fresh LB medium or infection of naïve BMM.

Quantitative reverse transcriptase PCR. Intracellular bacteria were harvested following 30 min infection of 5 x 10^7 BMM for RNA extraction (Qiagen RNAeasy mini kit). 500 ng of RNA was used to synthesize complementary DNA (cDNA) (QuantiTect RT kit, Qiagen). 0.5 μl of cDNA was used in qPCRs (SybrGreen PCR master mix, Applied biosystems) containing 0.2 μM gene-specific primers. The cycle threshold (Ct) value was determined as the number of cycles necessary to reach a significant increase in the emission intensity of the SybR Green reporter

dye. Data are represented as relative amounts of mRNA normalized to a 16S control.

Statistical analysis. Statistical differences were determined using a one-tailed Student *t*-Test on the means of at least three independent experiments. Probability values of less than 0.05, 0.01 and 0.005 were used to show statistically significant differences and are represented with *, ** or *** respectively.

Fig. S1. Identification of *Salmonella* in animal tissue-derived samples by Flow Cytometry. Flow cytometric detection of pre-induced bacterial cells carrying pFCcGi (constitutive mCherry, inducible GFP) in either inoculum (top panels), uninfected murine Peyer's Patches (middle panels) or released from infected Peyer's Patches (lower panels). Bacterial cells were identified by first gating on events with similar FSC and SSC properties to *Salmonella*, followed by a second gating on particles emitting high levels of red fluorescence. Green fluorescence dilution was analysed to measure bacterial replication.

Fig. S2. Log percentage survival after 24 h of cef treatment of LB medium-grown bacteria, or bacteria within C57 Bl6 or 129SV (*slc11a*+) BMM, IFN-γ activated or not, for 24 h or 30 min followed by transfer to LB medium. Data represent the mean ± SEM and were analysed by comparison with LB medium using a Student's *t* test. * $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

Fig. S3. Experimental design of assessment of metabolic activity and regrowth of persisters, analysed by flow cytometry. Bacteria pre-loaded with GFP were used to infect BMM for up to 72 h. Replicating bacteria gradually lost the GFP signal and were killed by exposure to cefotaxime. Non-replicating bacteria retained green fluorescence and their metabolic status was determined by addition of DsRed inducer (arabinose). After release from BMM and transfer to LB medium for 2 h, potential regrowth was detected by fluorescence dilution.

Fig. S4. Quantification of proportion of non-replicating bacteria showing metabolic activity upon addition of inducer (production of DsRed fluorescence; red bars) and resuming growth (indicated by fluorescence dilution; grey bars), from intracellular non-replicating cells in +/- IFN-γ activated 129SV (*slc11a*+) BMM over time. Data represent the mean ± SEM and were analysed using a Student's *t* test. * $P < 0.05$ and ***, $P < 0.001$.

Fig. S5. Efficiency of drugs used to inhibit BMM processes. (**A**) Localisation of bacteria after treatment of BMM with Latrunculin B (LatB) or Cytochalasin D (CytD) prior to infection and compared to untreated BMM. (**B**) Reduced lysosomal enzyme activity was quantified by measuring fluorescent cleavage product of Cathepsin B substrate Magic Red-RR in BMM, after treatment of BMM with NH_4Cl or Bafilomycin A1 (BafA1) and normalised to untreated BMM. Data represent the mean ± SEM.

Fig. S6. (A) Log percentage survival of LB medium-grown WT *Salmonella* or Δ*lon* mutant strain after 5 h of cefotaxime (cef) or ciprofloxacin (cip) treatment. (**B**) Log percentage survival of minimum medium-grown WT *Salmonella* or Δ*lon* mutant strain after 24 h of cef treatment. Data represent the mean ± SEM.

Fig. S7. (**A**) Fold increase in mRNA levels for each candidate toxin/antitoxin gene pair in WT (orange bars) or Δ*relAspoT* mutant (black bars) strains of *Salmonella* after 30 min internalization in BMM relative to levels in LB medium-grown bacteria. (**B**) Fold increase in mRNA levels of each candidate toxin gene in WT *Salmonella* following 30 min exposure to SHX (red bars) or pH shift (grey bars) from 7.0 to 4.5, relative to levels in LB medium-grown bacteria. Data represent the mean ± SEM.

Fig. S8. Fold replication of WT or single TA module mutant strains in BMM, measured by FD (*4*). Data represent the mean ± SEM and were analysed by comparison with WT *Salmonella* using a Student's *t* test. ** $P < 0.01$.

Fig. S9. Formation of macrophage-induced persisters caused by 30 min internalization in BMM, of WT or single TA module mutant strains of *Salmonella* obtained from independent mutagenesis experiment. Data represent the mean ± SEM and were analysed by comparison with WT *Salmonella* using a Student's *t* test. * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ns, not significant.

Fig. S10. Model depicting alternative fates of bacteria in response to vacuolar stress. Following phagocytosis, *Salmonella* is exposed to acidification and nutrient deprivation within the lumen of the SCV. These signals induce expression of virulence genes including those encoding the SPI-2 T3SS. They also lead, *via* the stringent response, to the activation of 14 TA modules whose up-regulation induces the formation of non-replicating persistent bacteria.

1

2

3

Table S1

S. Typhimurium 12023 strains		
Name	Description	Source or Reference
wild-type	12023 *S.* Typhimurium wild-type	NTCC
Δ*lon*	Δ*lon*::cm	This study
Δ*relA*/Δ*spoT*	Δ*relA*::km/Δ*spoT*::cm	(*7*)
Δ*relBE1*	Δ*relBE1*::km	This study
Δ*relBE2*	Δ*relBE2*::km	This study
Δ*relBE3*	Δ*relBE3*::km	This study
Δ*relBE4*	Δ*relBE4*::km	This study
Δ*relBE5*	Δ*relBE5*::km	This study
Δ*TA6*	Δ*TA6*::km	This study
Δ*TA8*	Δ*TA8*::km	This study
Δ*TA9*	Δ*TA9*::km	This study
Δ*shpAB*	Δ*shpAB*::km	This study
Δ*parDE*	Δ*parDE*::km	This study
Δ*higBA1*	Δ*higBA1*::km	This study
Δ*higBA2*	Δ*higBA2*::km	This study
Δ*vapBC*	Δ*vapBC*::km	This study
Δ*phd/doc*	Δ*phd/doc*::km	This study

Plasmids		
Name	Description	Source or Reference
pKD3	PCR template plasmid with Cm resistance cassette (cm^R)	(*1*)
pKD4	PCR template plasmid with Km resistance cassette (km^R)	(*1*)
pKD46	Plasmid encoding arabinose-inducible λ-Red recombinase	(*1*)
pFCcGi	*rpsM::mCherry* and P_{BAD}*::gfpmut3a* promoter fusions	(*3*)
pDiGi	P_{LAC}*::gfpmut3a* and P_{BAD}*::mCherry* promoter fusions	(*4*)
pDiGc	*rpsM::gfpmut3a* and P_{BAD}*::dsRed* promoter fusions in pBAD18	(*44*)

Table S1. List of strains and plasmids used in this study. Abbreviations: Carb, carbenicillin; Km, kanamycin; Cm, chloramphenicol

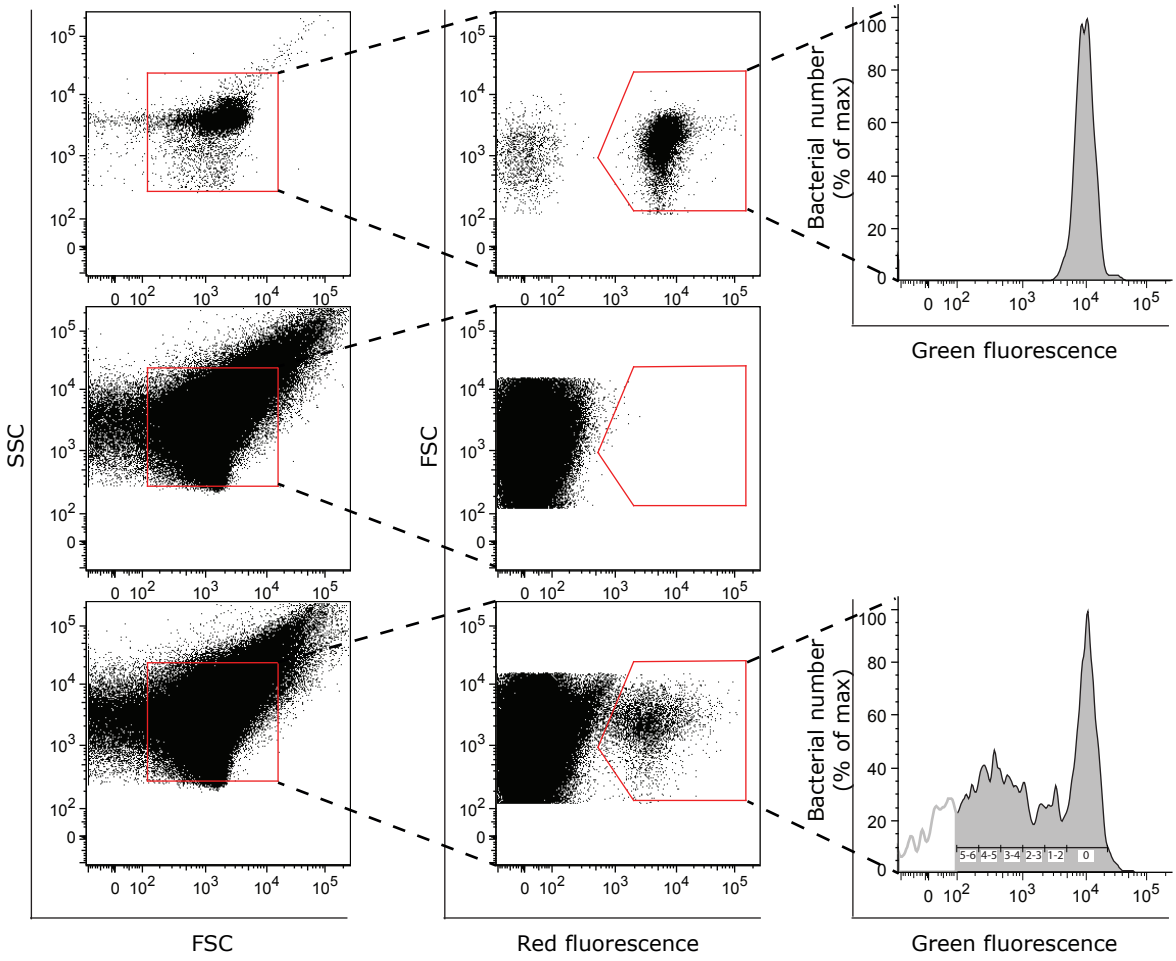
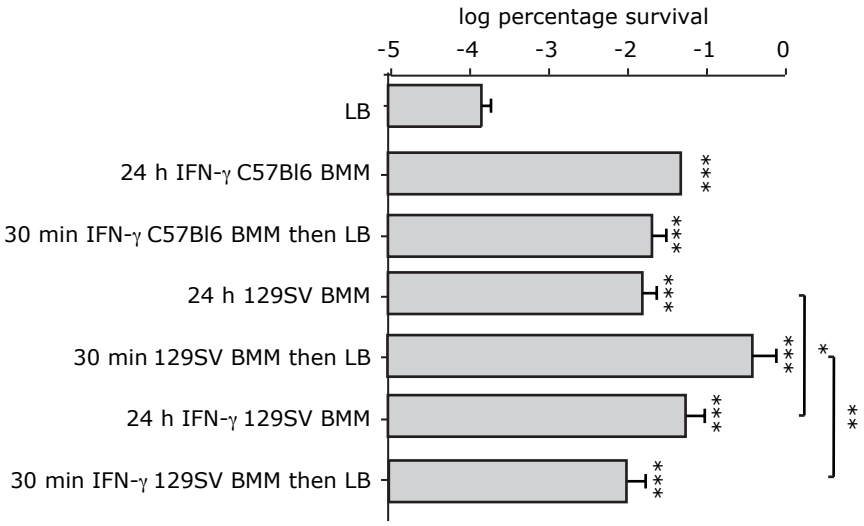

SSC
FSC
FSC
Red fluorescence
Bacterial number (% of max)
Green fluorescence
Bacterial number (% of max)
Green fluorescence
5-6
4-5
3-4
2-3
1-2
0

Fig. S1


log percentage survival
-5
-4
-3
-2
-1
0
LB
24 h IFN-γ C57Bl6 BMM
30 min IFN-γ C57Bl6 BMM then LB
24 h 129SV BMM
30 min 129SV BMM then LB
24 h IFN-γ 129SV BMM
30 min IFN-γ 129SV BMM then LB
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***
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***
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***
***
*
**

Fig. S2

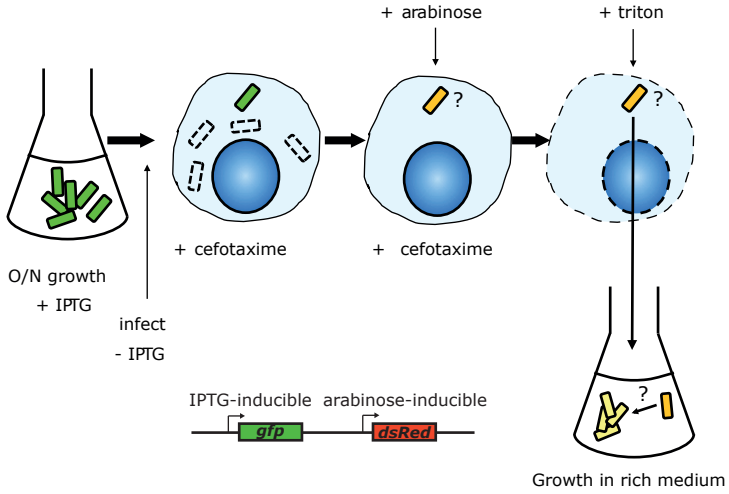
+ arabinose
+ triton
?
?
+ cefotaxime
+ cefotaxime
O/N growth
+ IPTG
infect
- IPTG
IPTG-inducible
arabinose-inducible
gfp
dsRed
?
Growth in rich medium

Fig. S3

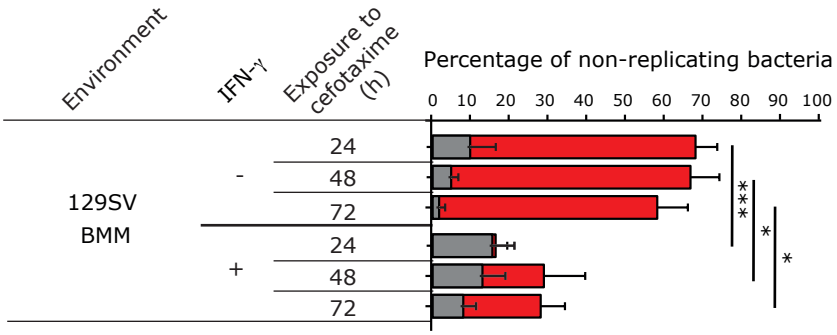
Environment
IFN-γ
Exposure to cefotaxime (h)
Percentage of non-replicating bacteria
0 10 20 30 40 50 60 70 80 90 100
129SV BMM
-
+
24
48
72
24
48
72
***
*
*

Fig. S4

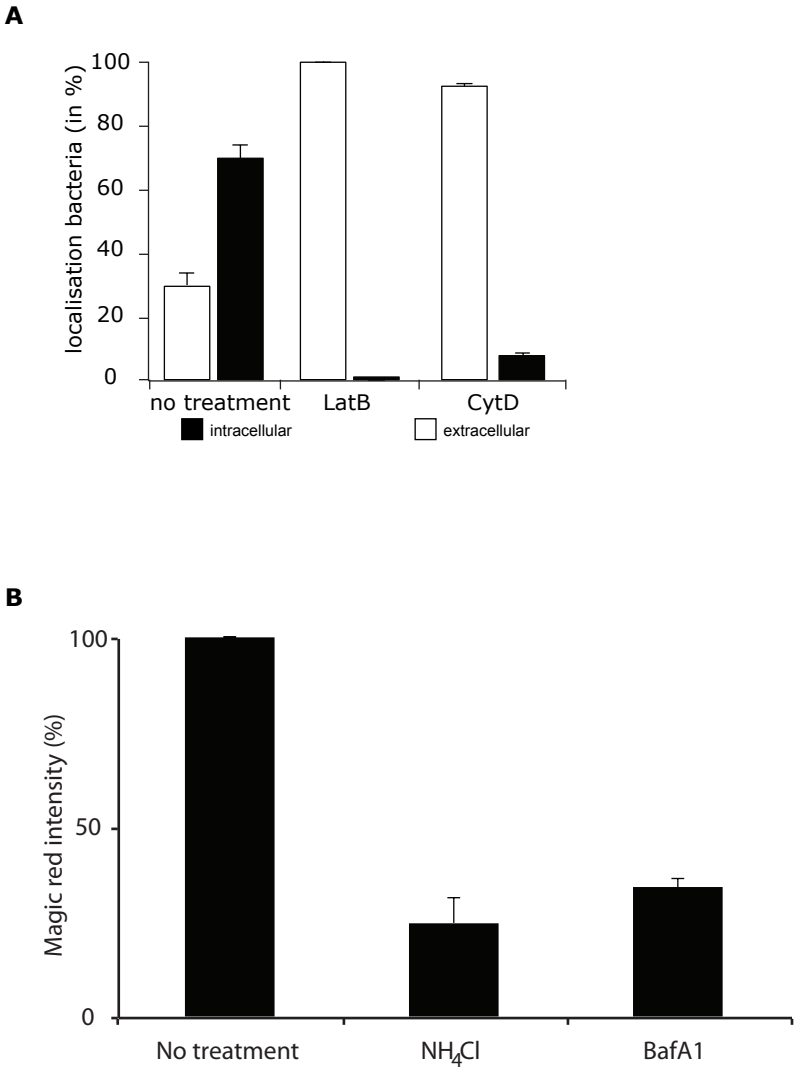
A
localisation bacteria (in %)
100
80
60
40
20
0
no treatment
LatB
CytD
intracellular
extracellular
B
Magic red intensity (%)
100
50
0
No treatment
NH4Cl
BafA1

Fig. S5

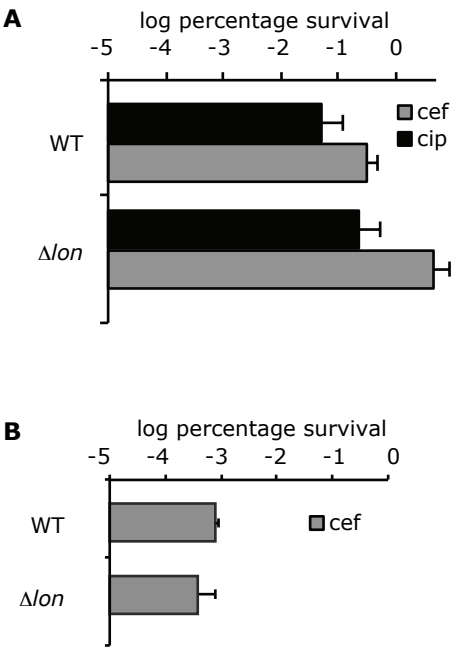
A
log percentage survival
-5
-4
-3
-2
-1
0
cef
cip
WT
Δlon
B
log percentage survival
-5
-4
-3
-2
-1
0
cef
WT
Δlon

Fig. S6

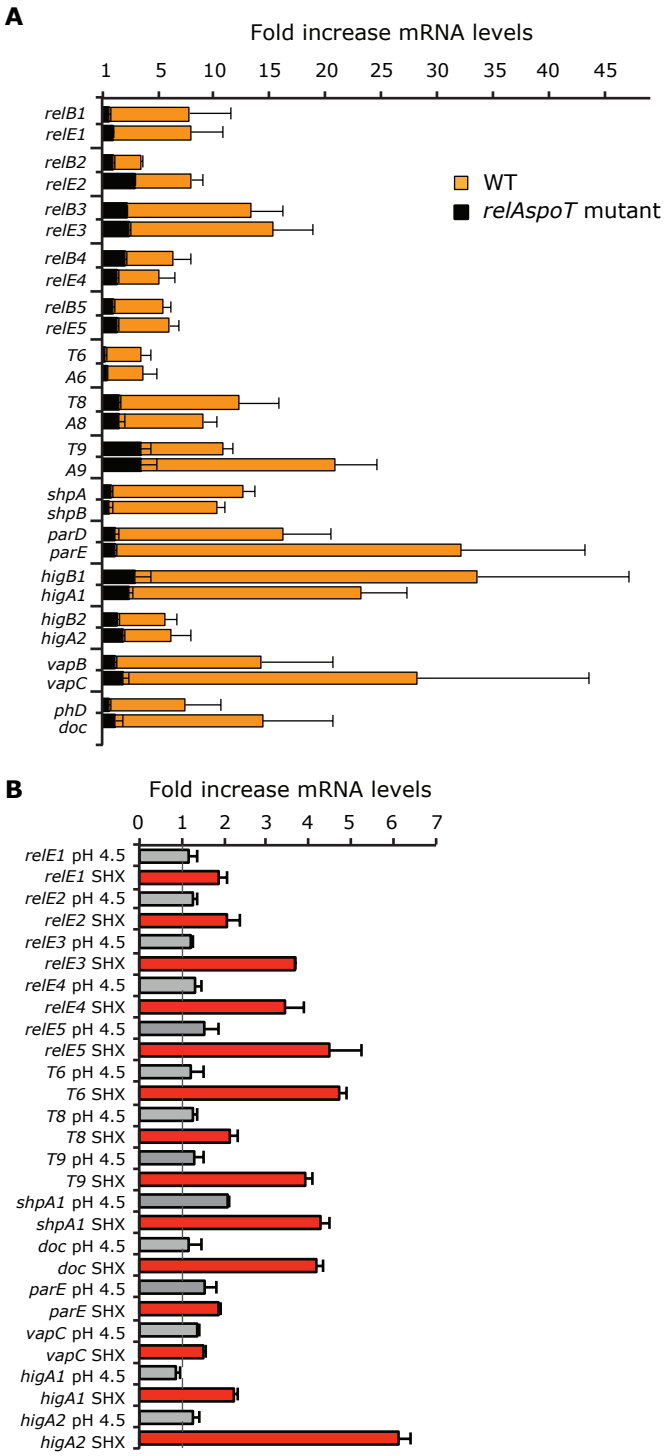
A
Fold increase mRNA levels
1
5
10
15
20
25
30
35
40
45
relB1
relE1
relB2
relE2
relB3
relE3
relB4
relE4
relB5
relE5
T6
A6
T8
A8
T9
A9
shpA
shpB
parD
parE
higB1
higA1
higB2
higA2
vapB
vapC
phD
doc
WT
relAspoT mutant
B
Fold increase mRNA levels
0
1
2
3
4
5
6
7
relE1 pH 4.5
relE1 SHX
relE2 pH 4.5
relE2 SHX
relE3 pH 4.5
relE3 SHX
relE4 pH 4.5
relE4 SHX
relE5 pH 4.5
relE5 SHX
T6 pH 4.5
T6 SHX
T8 pH 4.5
T8 SHX
T9 pH 4.5
T9 SHX
shpA1 pH 4.5
shpA1 SHX
doc pH 4.5
doc SHX
parE pH 4.5
parE SHX
vapC pH 4.5
vapC SHX
higA1 pH 4.5
higA1 SHX
higA2 pH 4.5
higA2 SHX

Fig. S7

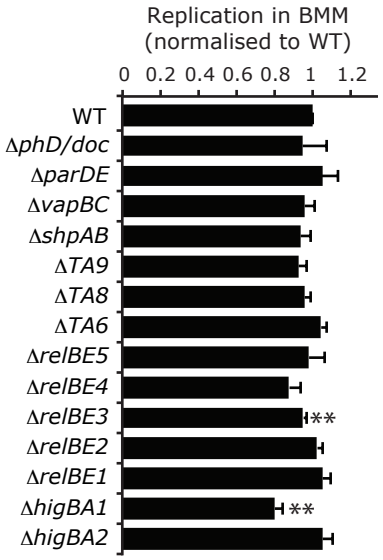
Replication in BMM
(normalised to WT)
0 0.2 0.4 0.6 0.8 1 1.2
WT
ΔphD/doc
ΔparDE
ΔvapBC
ΔshpAB
ΔTA9
ΔTA8
ΔTA6
ΔrelBE5
ΔrelBE4
ΔrelBE3
ΔrelBE2
ΔrelBE1
ΔhigBA1
ΔhigBA2
**
**

Fig. S8.

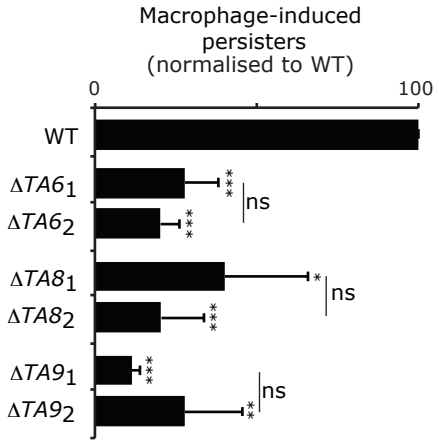
Macrophage-induced
persisters
(normalised to WT)
0
100
WT
ΔTA6₁
ΔTA6₂
ΔTA8₁
ΔTA8₂
ΔTA9₁
ΔTA9₂
***
***
ns
*
***
ns
***
***
ns

Fig. S9

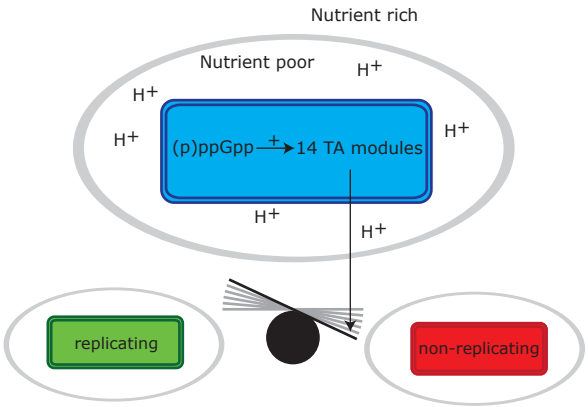
Nutrient rich
Nutrient poor
H+
H+
H+
H+
(p)ppGpp
+
14 TA modules
H+
H+
replicating
non-replicating

Fig. S10

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